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<b>(54) Title:</b> SERUM-FREE MEDIA FOR PRIMITIVE HEMATOPOIETIC CELLS AND METHODS OF USE THEREOF <b>(57) Abstract</b> <p>The invention provides compositions suitable for serum-free liquid culture, expansion, transduction, cryopreservation, etc. of human hematopoietic progenitor and stem cells.</p>			

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SERUM-FREE MEDIA FOR PRIMITIVE HEMATOPOIETIC CELLS AND METHODS  
OF USE THEREOF

The invention provide compositions suitable for serum-free culture, expansion, transduction, cryopreservation etc. of human hematopoietic progenitor and stem cells.

Mammalian hematopoietic cells provide a diverse range of physiological activities. These cells are divided into lymphoid, myeloid and erythroid lineages. The lymphoid lineage, comprising B cells and T cells, provides for the production of antibodies, regulation of the cellular immune system, detection of foreign agents in the blood, detection of cells foreign to the host, and the like. The myeloid lineage, which includes monocytes, granulocytes, megakaryocytes as well as other cells, monitors for the presence of foreign bodies, provides protection against neoplastic cells, scavenges foreign materials, produces platelets, and the like. The erythroid lineage provides the red blood cells, which act as oxygen carriers.

Despite the diversity of the nature, morphology, characteristics and function of hematopoietic cells, it is presently believed that these cells are derived from a single precursor cell population, termed "stem cells." Stem cells are capable of self-regeneration and can become lineage committed progenitors which are dedicated to differentiation and expansion into a specific lineage. As used herein, "stem cells" refers to hematopoietic cells and not stem cells of other cell types.

Stem cells constitute only a small percentage of the total number of hematopoietic cells. Hematopoietic cells are identifiable by the presence of a variety of cell surface

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"markers." Such markers can be either specific to a particular lineage or progenitor cell or be present on more than one cell type. Currently, it is not known how many of the markers associated with differentiated cells are also present on stem cells. One marker, which was previously indicated as present solely on stem cells, CD34, is also found on a significant number of lineage committed progenitors. U.S. Pat. No. 4,714,680 describes a population of cells expressing the CD34 marker. Characterizations and isolation of stem cells are reported in: Baum et al. (1992) Proc. Natl. Acad. Sci. USA 89:2804-2808; and Tsukamoto et al. U.S. Patent No. 5,061,620.

A pluripotent stem cell can be defined as follows:

(1) gives rise to progeny in all defined hematolymphoid lineages; and (2) limiting numbers of cells are capable of fully reconstituting a seriously immunocompromised host in all blood cell types and their progenitors, including the pluripotent hematopoietic stem cell, by self-renewal.

A highly purified population of stem cells is necessary for a variety of *in vitro* experiments and *in vivo* indications. For instance, a purified population of stem cells will allow for identification of growth factors associated with their self-regeneration. In addition, there may be as yet undiscovered growth factors associated with: (1) the early steps of dedication of the stem cell to a particular lineage; (2) the prevention of such dedication; and (3) the negative control of stem cell proliferation.

Stem cells find use in: (1) regenerating the hematopoietic system of a host deficient in any class of hematopoietic cells; (2) a host that is diseased and can be treated by removal of bone marrow, isolation of stem cells and treatment with drugs or irradiation prior to re-engraftment of stem cells; (3) producing various hematopoietic cells; (4) detecting and evaluating growth factors relevant to stem cell self-regeneration; and (5) the development of hematopoietic cell lineages and assaying for factors

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associated with hematopoietic development.

Stem cells are important targets for gene therapy, where the inserted genes promote the health of the individual into whom the stem cells are transplanted. In addition, the ability to isolate stem cells can serve in the treatment of lymphomas and leukemias, as well as other neoplastic conditions where the stem cells are purified from tumor cells in the bone marrow or peripheral blood, and reinfused into a patient after myelosuppressive or myeloablative chemotherapy. While stem cells are found in the bone marrow, they are preferable to bone marrow in "bone marrow transplantation." One reason for this is the presence of minimal residual disease, particularly leukemias, in bone marrow cells. By purifying stem cells away from the more differentiated and diseased cells, a patient has far less of a chance of relapse due to the presence of diseased cells in the transplant. A discussion of the presence of minimal residual disease (multiple myeloma) is provided in Billadeau et al. (1992) 80:1818-1824.

The growth and expansion of stem cells has been the subject of much research. See, e.g. Dexter et al. Long-Term Bone Marrow Culture (1984) Alan R. Liss, Inc. pp.57-96. The small numbers of stem cells obtained from any one source and the primitive nature of the cells has led to a great deal of difficulty in maintenance and expansion of the cells. Stem cells used for in vitro purposes can be cultured in serum-containing media; however, for clinical use, cells which are to be returned to a patient must be maintained in a defined medium which is typically serum-free, although autologous serum may be used in some cases. Serum is derived from a variety of animal sources, usually bovine. Preparations of serum vary from batch to batch. Some batches have been shown to contain substances inhibitory to cell growth. See, e.g. Drouet et al. (1989) Br. J. Haemat. 73:143-147. Serum-free media have been developed for a variety of cell types. Deslex et al. describe a chemically defined serum-free medium for

differentiation of rat adipose precursor cells. (1987) Exp. Cell Res. 168:15-30. U.S. Patent No. 5,397,706 describes a serum-free medium that promotes differentiation of hematopoietic cells.

To date, stem cells have routinely been cultured on a stromal cell layer, in a small volume of serum-containing media. In order to provide therapeutically acceptable stem cell compositions, the cells ideally are cultured, stored and preserved without stromal cells and without serum. The advantages of and attempts to formulate serum-free cell culture are reviewed in Barnes et al. (1980) Cell 22:649-655.

Preferably, due to the small number of stem cells available, the serum-free media allows expansion of cells while retaining a primitive (CD34<sup>+</sup>) phenotype. The growth of bone marrow cells in the absence of stromal cells and serum has been described by Teofili et al. (1992) Ann. Hematol. 65:22-25. The culture allowed normal hematopoiesis. As discussed above, stem cells are preferable to bone marrow in a variety of therapeutic indications. Several serum-free media have been devised for stem cell culture. Cormier et al. (1991) Growth Factors 4:157-164; Pointing et al. (1991) 4:165-173; U.S. Patent No. 5,397,706; WO 92/18615; WO95/00632; and WO 95/02685. At least one medium, Hematopoietic Stem Cell-SFM, is available commercially (GibcoBRL, Grand Island, NY).

The present invention provides compositions of serum-free media based on a basal medium with various additives. The additives include effective amounts of at least one of the following: a peptone, a protease inhibitor and a pituitary extract. The additives can also include effective amounts of at least one of the following: human serum albumin or plasma protein fraction, heparin, a reducing agent, insulin, transferrin and ethanolamine, low density lipoprotein or human cholesterol concentrate, linoleic acid, oleic acid, vitamin A, vitamin E, superoxide dismutase, ascorbic acid, putrescine, vitamin B12 and a steroid hormone.

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Compositions comprising the media and hematopoietic cells are also provided. Preferably, the cells are progenitor and stem cells. Compositions for cryopreservation are also provided and include the media, a cryopreservative and hematopoietic cells.

Figure 1 is a bar graph depicting total cell expansion (clear area) and maintenance of the CD34<sup>+</sup> phenotype (hatched area) of adult bone marrow (ABM) CD34<sup>+</sup>Thy-1<sup>-</sup>LIN<sup>-</sup> cells in the media indicated for six days in 5% O<sub>2</sub>. In Figure 1 PIT stands for bovine pituitary extract.

Figure 2 is a bar graph depicting total cell expansion (clear area) and maintenance of the CD34<sup>+</sup> phenotype (hatched area) of mobilized peripheral blood (MPB) CD34<sup>+</sup>Thy-1<sup>-</sup>LIN<sup>-</sup> cells in the media indicated for five days (2A) and seven days (2B).

Figure 3 is a graph depicting fold expansion of MPB CD34<sup>+</sup>Thy-1<sup>-</sup>LIN<sup>-</sup> cells maintained for thirteen days in SSP15 (open circles), SSP16 (closed circles) and IMDM/10%FBS (squares).

Figure 4 is a bar graph depicting total cell expansion (clear area) and maintenance of the CD34<sup>+</sup> phenotype (hatched area) of ABM CD34<sup>+</sup>Thy-1<sup>-</sup>LIN<sup>-</sup> cells in the indicated media for five days. The cells were initially inoculated at 2000 cells/100  $\mu$ L (4A) or 1400 cells/100  $\mu$ L (4B). Total growth is shown by the clear area. CD34<sup>+</sup> growth is shown by the hatched area.

Figure 5 is a bar graph depicting total cell expansion (clear area) and maintenance of the CD34<sup>+</sup> phenotype (hatched area) of ABM CD34<sup>+</sup>Thy-1<sup>-</sup>LIN<sup>-</sup> cells in the indicated media for five days in 5% O<sub>2</sub> (5A) and 20% O<sub>2</sub> (5B).

Figure 6 is a bar graph depicting total cell expansion (clear area) and maintenance of the CD34<sup>+</sup> phenotype (hatched area) of ABM CD34<sup>+</sup>Thy-1<sup>-</sup>LIN<sup>-</sup> cells derived from two different individuals, #11281 (6A) and #11279 (6B) and incubated for six days in the indicated media.

The present invention provides serum-free media for use in stem cell manipulation. Manipulations of stem cells include, but are not limited to, expansion, cryopreservation, shipping, processing and transduction. The cells can be grown in the absence of stromal cells which makes them particularly useful for human immune therapy. The media are described herein and have been shown to be more effective than standard IMDM plus 10 percent fetal calf serum in stem cell expansion and maintenance as measured by the presence of the CD34<sup>+</sup> phenotype.

The growth of stem cells in serum-free conditions were developed especially for expanding pluripotent stem cell populations to generate a cell population enriched in stem and progenitor cells for re-infusion into a patient. Such an expanded cell population provides a desirable graft for providing both short-term and long-term hematopoietic recovery to a patient after, e.g. myelosuppressive or myeloablative chemotherapy. In addition, optimization in the cells nutritional environment aids in maintaining cell viability during a variety of manipulations. To provide a serum-free environment, serum components that are potentially active on cultured cells need to be replaced by defined components. The hundreds of components found in sera can be grouped into the following categories: nutrients, hormones, binding proteins, attachment factors, enzymes and inhibitors. Each category contains numerous and varied components. For instance, the following subdivisions can be made. It should be noted that each subdivision contains numerous components. Nutrients contain vitamins and related compounds; lipoproteins; other serum lipids; trace elements; and other nutrients such as polyamines, amino acids, nucleosides, etc. Hormones contain pancreatic hormones; peptide growth factors; steroid hormones; prostaglandins/thromboxanes/leukotrienes/prostacyclin; pituitary hormones; thyroid/parathyroid hormones; and leukocyte products. Binding proteins include those that bind to vitamins; lipids; trace elements; peptide hormones;



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steroids; and toxins. Attachment factors include fibronectin; serum spreading factors; thrombospondin; and fibrinogen. Enzymes and inhibitors include thrombin; catalase; superoxide dismutase; protease inhibitors; other clotting/complement-related enzymes; and kininogenases/kinins.

Primitive hematopoietic cells are often referred to as stem cells and progenitor cells; as used herein "stem cells" means a population of hematopoietic cells containing stem cells and/or progenitor cells. For instance, cells purified on the basis of expression of the cell surface protein CD34 contain stem cells and a large percentage of progenitor cells. Preferably, the cells are human but can also be of animal origin. Methods of purifying stem cells are known in the art. For instance, stem cell purification is described in United States Patent Nos. 5,409,813 and 5,061,620.

The basal medium can be IMDM (JRH Biosciences, KS) which can be used as obtained from the manufacturer and used according to the manufacturer's instructions or prepared based on published amounts of individual components. Table 1 provides the published concentrations of IMDM, the preferred composition of the basal medium (hereinafter referred to as "SSP"), and the ranges of concentrations of substances suitable for use herein (hereinafter "basal medium"). As described herein, SSP is the preferred embodiment although basal and IMDM media are suitable for use herein. Thus, although the phrase "SSP" may be used to describe the embodiments of the invention, the phrase includes the various media included in IMDM and basal medium. In Table 1 the amounts of ingredients given are in grams per liter final concentration.

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Table 1

components	IMDM	SSP	basal medium	
arginine HCl	0.084	0.1008	0.0504	- 0.1512
choline chloride	0.004	0.0048	0.0024	- 0.0072
histidine HCl H <sub>2</sub> O	0.042	0.0504	0.0252	- 0.0756
isoleucine	0.105	0.126	0.063	- 0.189
leucine	0.105	0.126	0.063	- 0.189
lysine HCl	0.146	0.1752	0.0876	- 0.2628
methionine	0.03	0.036	0.0072	- 0.0432
phenylalanine	0.066	0.0792	0.0396	- 0.1188
serine	0.042	0.0504	0.0252	- 0.0756
threonine	0.095	0.114	0.057	- 0.171
tryptophan	0.016	0.0192	0.0096	- 0.0288
tyrosine-2Na	0.1038	0.12456	0.06228	- 0.18684
valine	0.094	0.1128	0.0564	- 0.1692
biotin	0.000013	1.56 x 10 <sup>-5</sup>	0.078	- 2.28 x 10 <sup>-5</sup>
pantothenic acid	0.004	0.0048	0.0024	- 0.0072
niacinamide	0.004	0.0048	0.0024	- 0.0072
thiamine HCl	0.004	0.0048	0.0024	- 0.0072
riboflavin	0.0004	0.00048	0.00024	- 0.00072
folic acid	0.004	0.0048	0.0024	- 0.0072
potassium chloride	0.33	0.396	0.198	- 0.594
calcium chloride 2H <sub>2</sub> O	0.219	0.2628	0.1314	- 0.3942
phenol red	-	-	-	-
pyruvic acid Na	0.11	0.132	0.066	- 0.198
asparagine H <sub>2</sub> O	0.0284	0.03408	0.01704	- 0.05112
proline	0.04	0.048	0.024	- 0.072
vitamin B12	0.000013	1.56 x 10 <sup>-5</sup>	0.078	- 2.28 x 10 <sup>-5</sup>
alanine	0.025	0.03	0.015	- 0.045
aspartic acid	0.03	0.036	0.018	- 0.054
glutamic acid	0.075	0.09	0.045	- 0.135
glycine	0.03	0.036	0.018	- 0.054
myo-inositol	0.0072	0.00864	0.00432	- 0.01296
lipoic acid	0.000105	0.000126	0.000063	- 0.000189
sodium selenite	0.000017	2.04 x 10 <sup>-5</sup>	1.02	- 3.06 x 10 <sup>-5</sup>
cystine 2 HCl	0.09124	0.109488	0.054744	- 0.164232
2-mercaptoethanol	0.0036	0.00432	0.00216	- 0.00648
HEPES	5.958	-	-	-
magnesium sulfate	0.09767	0.117204	0.058602	- 0.175806
potassium nitrate	0.000076	9.12 x 10 <sup>-5</sup>	4.56	- 13.68 x 10 <sup>-5</sup>
sodium phosphate monobasic	0.109	0.1308	0.0654	- 0.1962
pyroxidal	0.004	0.0048	0.0024	- 0.0072
glucose	4.5	4.5	2.25	- 6.75
sodium bicarbonate	3.024	3.024	1.512	- 4.536
glutamine	0.584	0.7008	0.3504	- 1.0512
dl-alpha tocopherol	0.00018	0.000216	0.000108	- 0.000324
sodium chloride	4.505	-	-	-
sodium chloride use to adjust osmolality to 280 mOsm		4-5 G / L	-	-

To the basal media is added a number of components to provide optimal nutrients and factors suitable for stem cell growth and maintenance. These additives should be present in effective amounts. "Effective" amounts are those which have a beneficial effect on a cell parameter without exhibiting a

deleterious effect. Various cell parameters to be monitored include, but are not limited to, viability, expansion, and maintenance of primitive phenotype during cryopreservation, shipping, processing and transduction. Methods of expanding, preserving, etc. stem cells are known in the art and are not described in detail herein. Stem cell expansion is described for instance in U.S. Patent No. 5,409,825.

In particular at least one of the following additives is added to the medium: a peptone, a protease inhibitor and a pituitary extract.

Preferably, the peptones are autoclavable. Sources of autoclavable peptones include, but are not limited to, meat peptone, soybean peptone and Primatone™ (Primatone™ is the trade name for a mix of horse, pork and beef peptones). Preferably, the peptone is meat peptone. Meat peptone is provided as powder from the manufacturer (Sigma) and made into a stock solution at 10% and autoclaved prior to use. The stock solution is then added to the base medium in amount of from about 5-35 ml/L (0.05 to 0.35%). Preferably, the stock solution added is about 20 ml/L (0.2%). Soy bean peptone (Sigma) is effective in a range of from 0.1% to 1.0%. Soy bean peptone is not as effective as meat peptone. Primatone™ (Sigma) is effective in a range of from 0.1% to 1.0%. Primatone™ is not as effective as meat peptone. Autoclavable peptones further enhance amino acids, salts and dipeptide concentrations.

Suitable protease inhibitors include any which are cell compatible. Protease inhibitors minimize apoptosis. These include, but are not limited to, aprotinin, superoxide dismutase (SOD) and catalase. Preferably, the protease inhibitor is aprotinin. More preferably, the aprotinin is recombinant (Miles). Preferably, the aprotinin is present in an amount of from 2,000 to 20,000 KIU units in the final solution. More preferably, the aprotinin is present in an amount of 10,000 KIU units.

Suitable pituitary extracts include any which are cell

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compatible and exert a beneficial effect on the cells. Preferably the pituitary extract is bovine (Collaborative Biomedical Products, Bedford, MA). More preferably, the pituitary extract is human. Bovine pituitary extract is present in an amount from 3 mg/L to 75 mg/L. More preferably, the pituitary extract is present in an amount of 30 mg/L. This concentration has been found to be beneficial for expansion of stem cells. However, as this extract is not defined and is a bovine product, it may not be clinically acceptable for human use and thus is preferably not added if the stem cells are to be reintroduced to a human. Suitable substitutes include one or more of the purified factors found in pituitary extract including, e.g., activin, inhibin, heregulin and growth hormone.

Optionally, effective amounts of at least one of the following can also be added. These include, but are not limited to, human serum albumin or plasma protein fraction, heparin, a reducing agent, insulin, transferrin and ethanolamine, low density lipoprotein or human cholesterol concentrate, linoleic acid, oleic acid, vitamin A, vitamin E, superoxide dismutase, ascorbic acid, putrescine, vitamin B12 and a steroid hormone.

Serum albumin is preferably added in an effective amount. Suitable serum albumins include, but are not limited to, human, bovine and equine. Preferably, the serum albumin is human. Alternatively, autologous human serum can be used. Human serum albumin ("HSA") solution is provided as a 25% solution for I.V. administration, USP (Alpha Therapeutic Corp., Los Angeles, CA). Preferably, human albumin solution is present in an amount from 40-120 ml of the 25% solution per liter of medium (1% to 3% final concentration). More preferably, human albumin solution is present in an amount of 80 ml of the 25% solution per liter of medium (2% final concentration). Optionally, plasma protein fraction can replace human serum albumin. Plasma protein fraction, human, USP (Alpha) is provided in a 5% solution. Preferably, plasma

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protein fraction is present in an amount of from 1% to 4% final concentration. Plasma protein fraction can be as effective or more effective than HSA but is difficult to work with.

Anti-clotting compounds, preferably heparin, can also be added. Heparin is provided as heparin sodium solution, USP, injection grade (Lyphomed, Deerfield, IL). The stock solution is 20,000 USP units/ml. Preferably, heparin is present in a final concentration from 5,000 to 40,000 USP units. More preferably, heparin is present in a final concentration of 20,000 USP units.

The reducing agent can be any cell compatible reducing agent known in the art, including, but not limited to thiols, aminoguanidine, 3-hydroxybenzylhydroxylamine or low  $O_2$  tension. Preferably, the reducing agent is  $\beta$ -mercaptoethanol.  $\beta$ -mercaptoethanol (Sigma) is diluted to a 50 mM stock solution. Preferably,  $\beta$ -mercaptoethanol is present in a final concentration of from 50 to 300  $\mu$ M. More preferably,  $\beta$ -mercaptoethanol is present in a final concentration of 100  $\mu$ M.

The insulin can be obtained from any known source. Preferably the insulin is of the same source as the cells. In the case of use with human cells, the insulin is preferably human recombinant. The zinc salt of human recombinant insulin is available from Miles, Inc., Kankakee, IL. The stock solution is 2,000 mg/L. Preferably, the final concentration of insulin is from 2 to 100 mg/L medium. More preferably, the concentration of insulin is 20 mg/L.

Transferrin can be obtained from any known source. Preferably, for use with human cells, the transferrin is human (Miles, partially iron saturated). The stock solution provided is 4,000 mg/L. Preferably, the transferrin is present in an amount of 2-400 mg/L. More preferably, the transferrin is present in an amount of 40 mg/L.

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Ethanolamine or phosphoethanolamine are also added in an effective amount. Ethanolamine can be obtained from any known source. Ethanolamine (Sigma) stock solution is provided at 660 mg/L. Preferably, the ethanolamine is present in amount of 2.2 to 11.0 mg/L. More preferably, the ethanolamine is present in an amount of 6.6 mg/L. Typically, insulin, transferrin and ethanolamine are combined into one solution (ITE).

Low density lipoprotein (LDL) can be from any known source. Preferably, the LDL is human when the medium is to be used with human cells. LDL (Sigma) is preferably present in an amount from 1.0 to 10.0 mg/L. More preferably, the LDL is present in an amount of 2.5 mg/L. Optionally, human cholesterol concentrate can be used in addition to or in place of LDL. Preferably the cholesterol concentrate (Miles) is present in an amount from 2.13 mg/L to 340.48 mg/L. Preferably, HDL is not added as it is inhibitory to stem cell expansion and maintenance.

Linoleic acid can be from any known source. Preferably, the linoleic acid (Sigma) is provided in a stock solution of 0.1 M. Oleic acid can be from any known source. Preferably, the oleic acid (Sigma) is provided in a stock solution of 0.1 M. Vitamin A can be from any known source. Preferably, the vitamin A (Sigma) is provided in a stock solution of 0.1 M. Vitamin E can be from any known source. Preferably, the vitamin E (Sigma) is provided in a stock solution at 0.1 M. Linoleic acid, oleic acid, vitamin A and vitamin E are not soluble in water. They are dissolved in 100% ethanol at a concentration of 0.1 M. All are added at 200  $\mu$ l/L of the medium for a final concentration of  $2 \times 10^{-5}$  M.

Preferably, the medium contains an antioxidant. Suitable antioxidants are any known in the art and include, but are not limited to superoxide dismutase and catalase. Preferably, the antioxidant is superoxide dismutase (Sigma). Preferably the superoxide dismutase is present in an amount of from 2,000 to 20,000 units per liter of media. More preferably, the superoxide dismutase is present in an amount of 10,000 units

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per liter of media.

The media can also contain ascorbic acid. The ascorbic acid can be from any known source. Ascorbic acid (Sigma) is provided in a stock solution at  $10^{-2}$  M. Preferably, the ascorbic acid is present in an amount of from  $10^{-3}$  to  $10^{-7}$  M final concentration. More preferably, the ascorbic acid is present in an amount of  $10^{-5}$  M final concentration.

Polyamines can also be added. Suitable polyamines include, but are not limited to, putrescine, spermine and spermidine. Preferably, the polyamine is putrescine. The putrescine can be from any known source. Putrescine (Sigma) is provide in a stock solution of  $10^{-4}$  M. Preferably, the putrescine is present in an amount of from  $10^{-5}$  to  $10^{-9}$  M final concentration. More preferably, the putrescine is present in an amount of  $10^{-7}$  M final concentration.

Vitamin B12 can be from any known source. Vitamin B12 (Sigma) is provided in a stock solution of  $10^{-3}$  M. Preferably, the vitamin B12 is present in an amount of  $10^{-4}$  to  $10^{-8}$  M final concentration. More preferably, vitamin B12 is present in an amount of  $10^{-6}$  M final concentration.

Desirably, the media can contain at least one steroid hormone. Steroid hormones can be obtained from any known source and include, but are not limited to, progesterone, estrogen, androgen, testosterone and physiologically effective analogs thereof. Testosterone (Sigma) is provided in a stock solution at  $10^{-6}$  M final concentration. Preferably, testosterone is present in an amount of from  $10^{-6}$  to  $10^{-12}$  M final concentration. More preferably, testosterone is present in an amount of  $10^{-9}$  M final concentration. Progesterone (Sigma) is provided in a stock solution at  $10^{-7}$  M final concentration. Preferably, progesterone is present in an amount of  $10^{-7}$  to  $10^{-13}$  M. More preferably, progesterone is present in an amount of  $10^{-10}$  M final concentration.

Various other additives known in the art of cell culture can also be added. These include, but are not limited to, interleukins, cytokines and growth factors. Leukemia

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inhibitory factor (LIF) has been described for use in propagation of stem cells. PCT/US93/01852. WO 93/08268 describes the use of stem cell factor (SCF, also called c-kit ligand (KL) mast cell growth factor (MGF)), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and interleukin-6 (IL-6). Preferably, the added factors include, but are not limited to, IL3, IL6, c-kit ligand, GM-CSF and flk2/flt3 ligand. Brandt et al. describe a variety of factors and their use. (1994) Blood 83:1507-1514. See also U.S. Patent No. 5,409,825.

The osmolality of the solution is titrated from 232 mOsm to 377 mOsm with NaCl. Preferably the osmolality is from 260 to 320 mOsm. More preferably, the osmolality is 290 mOsm.

Preferably, the pH of the solution is adjusted to about 6.9 prior to filtration. Suitable buffers include, but are not limited to, sodium bicarbonate, HEPES, phosphate and lactate.

Various other components can optionally be added to the medium. For instance, Ex-cyte IV (Human growth enhancement media supplement) (Miles) was titrated from 1.05 mg/L to 84.0 mg/L and found to produce variable results. It can be useful under certain circumstances. Preferably, antibiotics are not added to the medium. However, certain antibiotics can be beneficial. It has been found that polymyxin B sulfate (Sigma), titrated from 10 µg/L to 100 µg/L causes a slight decrease in cell expansion but can be useful for its antibiotic properties. Therefore, antibiotics including, but not limited to, polymyxin B can be added provided they do not cause more than a slight decrease in cell expansion etc.

In addition, some additives were found to be inhibitory. Fetuin (bovine, Sigma) was titrated from 25 mg/L to 400 mg/L was found to provide no benefit to cell growth/expansion. Preferably, the medium does not contain fetuin.

Several preferred combinations of media have been formulated. These are designated SSP12, SSP14, SSP15 and



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SSP16. These media have the following formulations, where SSP is the basal medium and has the formulation described in Table 1.

SSP12: 2% HSA, 20 IU/ml heparin, 2 mM 2-mercaptoethanol,  $1 \times 10^{-5}$  M each of insulin, transferrin and ethanolamine.

SSP14: in addition to components of SSP12, includes 2.5 µg/L LDL, 0.2% meat peptone,  $2 \times 10^{-5}$  M linoleic acid,  $2 \times 10^{-5}$  M oleic acid,  $2 \times 10^{-5}$  M vitamin A,  $2 \times 10^{-5}$  M vitamin E, 10 KIU/mL aproptinin and 30 µl/mL pituitary extract. SSP15: in addition to the components of SSP14, contains also 10 units/mL superoxide dismutase and  $10^{-5}$  M vitamin C. SSP16: in addition to the components of SSP15, contains also  $10^{-6}$  M putrescine, vitamin B12, progesterone and testosterone at the preferred concentrations provided above.

The invention also encompasses compositions containing the media described above and stem cells. The stem cells can be of any degree of purity provided they are enriched at least in progenitor cells and more primitive cells.

The invention also encompasses improved compositions for use in cryopreservation of stem cells. The compositions include the SSP media described above and a cryopreservative such as DMSO. The compositions can further contain stem cells. The stem cells can be of any degree of purity provided they are enriched at least in progenitor cells and more primitive cells. The composition can further contain Hetastarch™, pentastarch or methylcellulose. Preferably, Hetastarch™ is present in an amount from 1% to 4%. More preferably, the Hetastarch™ is present in an amount of 1.4%.

The cells obtained as described above can be used immediately or frozen at liquid nitrogen temperatures and stored for long periods of time, being thawed and capable of being reused. The cells will usually be stored in 7%-7.5% DMSO, 1.4% hetastarch, 4% HSA, which is designated SSP cryopreservation medium.

The invention also comprises methods of manipulating stem cells in the presence of the serum-free media described

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herein. In the case of stem cell expansion, the cultures are preferably maintained, at least initially, in a hypoxic environment, although the use of antioxidants and reducing agents in the media formulation may limit the need to grow the cells in low oxygen environments. The use of hypoxic stem cell culture was found to increase differentiation of the cells in the presence of cytokines. Ishikawa et al. (1988) 40:126-129. The cells are preferably grown in a bioreactor.

Use of the improved serum-free media formulations described herein has been found to cause stem cells, normally quiescent, to enter cell-cycle more rapidly in culture. Therefore, the time needed to obtain an optimal graft of stem/progenitor cells is reduced. In addition, improved transduction of the stem cells can be achieved. Retroviral vectors are known to require cell division for integration into the target cell genome. The media formulations of the present invention improve cell viability and the rate of cell cycling, thereby improving overall transduction efficiency.

Stem cells can be isolated from any known source of stem cells, including, but not limited to, bone marrow, both adult and fetal, mobilized peripheral blood (MPB) and umbilical cord blood. The use of umbilical cord blood is discussed, for instance, in Issaragrishi et al. (1995) N. Engl. J. Med. 332:367-369. Initially, bone marrow cells can be obtained from a source of bone marrow, including but not limited to, ilium (e.g. from the hip bone via the iliac crest), tibia, femora, spine, or other bone cavities. Other sources of stem cells include, but are not limited to, embryonic yolk sac, fetal liver, and fetal spleen.

For isolation of bone marrow, an appropriate solution can be used to flush the bone, preferably with the media described herein, or with other serum-free solutions including, but not limited to, salt solution, which can be supplemented with other naturally occurring factors, in conjunction with an acceptable buffer at low concentration, generally from about 5-25 mM. Convenient buffers include, but are not limited to,

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HEPES, phosphate buffers and lactate buffers. Otherwise bone marrow can be aspirated from the bone in accordance with conventional techniques.

Various techniques can be employed to separate the cells by initially removing cells of dedicated lineage. Monoclonal antibodies are particularly useful for identifying markers associated with particular cell lineages and/or stages of differentiation. The antibodies can be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the retention of viability of the fraction to be collected. Various techniques of different efficacy can be employed to obtain "relatively crude" separations. Such separations are where up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present not having the marker can remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Procedures for separation can include, but are not limited to, magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, e.g., plate, or any other convenient technique.

The use of separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rhodamine123 and DNA-binding dye Hoechst 33342).

Techniques providing accurate separation include, but are not limited to, FACS, which can have varying degrees of sophistication, e.g., a plurality of color channels, low angle

and obtuse light scattering detecting channels, impedance channels, etc.

In a first separation, typically starting with about  $1 \times 10^8$ - $9$ , preferably at about  $5 \times 10^8$ - $9$  cells, an antibody specific for stem cells can be labeled with one fluorochrome, while the antibodies for the various dedicated lineages can be conjugated to at least one different fluorochrome. While each of the lineages can be separated in a separate step, desirably the lineages are separated at the same time as one is positively selecting for stem cells. The cells can be selected against dead cells, by employing dyes associated with dead cells (including but not limited to, propidium iodide (PI)). Preferably, the cells are collected in a serum-free medium.

The purified stem cells have low side scatter and low to medium forward scatter profiles by FACS analysis. Cytospin preparations show the enriched stem cells to have a size between mature lymphoid cells and mature granulocytes. Cells can be selected based on light-scatter properties as well as their expression of various cell surface antigens.

While it is believed that the particular order of separation is not critical to this invention, the order indicated is preferred. Preferably, cells are initially separated by a coarse separation, followed by a fine separation, with positive selection for stem cells.

The purified stem cells can be used for the treatment of genetic diseases. Thus, the stem cells can be used in treatment of genetic diseases associated with hematopoietic cells by genetic modification of autologous or allogeneic stem cells to correct the genetic defect. The present invention thus encompasses compositions for transduction of stem cells comprising stem cells and the serum-free media. The cells can be used for example, diseases including, but not limited to,  $\beta$ -thalassemia, sickle cell anemia, adenosine deaminase deficiency, recombinase deficiency, recombinase regulatory gene deficiency, etc. can be corrected by introduction of a

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wild-type gene into the stem cells, either by homologous or random recombination. Other indications of gene therapy are introduction of drug resistance genes to enable normal stem cells to have an advantage and be subject to selective pressure during chemotherapy. Suitable drug resistance genes include, but are not limited to, the gene encoding the multidrug resistance (MDR) protein.

The following examples are provided to illustrate but not limit the invention.

The results show that, in every case, the cytokine-supplemented SSP serum-free media formulations of the present invention provide better CD34<sup>+</sup> cell expansion, and usually better total cell expansion than that obtained using standard IMDM with serum.

#### Example 1

##### Materials and Methods

##### Cell Isolation and Manipulation.

Sorted CD34<sup>+</sup>Thy1<sup>+</sup>Lin<sup>-</sup> cells from either adult bone marrow (ABM) or mobilized peripheral blood (MPB) were used for each experiment. The culture conditions varied with each experiment as described in the separate examples.

For ABM, fresh 20 mL of bone marrow was isolated by aspiration of the iliac crest from human normal volunteers from Stanford University Medical Center (Palo Alto, CA) or Scripps Research Institute (La Jolla CA). Marrow was separated by taking the mononuclear cell fraction following a Ficoll-Perque separation, positive-selected for CD34<sup>+</sup> cells according to the method described by Sutherland et al. (1992) Exp. Hematol. 20:590. Briefly, cells were resuspended in staining buffer (SB) (HBSS containing 10 mM HEPES, 2% heat-inactivated FCS) at  $5 \times 10^7$  cells/mL. QBEND10 (anti-CD34) (Amac, Westbrook, ME) was added at 1/100 dilution, and cells incubated on ice for 30 min. Cells were then washed in SB with a FCS underlay, and resuspended at  $4 \times 10^7$ /mL in SB. An equal volume of washed Dynal sheep anti-mouse IgG<sub>1</sub>Fc magnetic

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beads (Dynal, Oslo, Norway), was added at a 1:1 bead to cell ratio, to give a final cell concentration of  $2 \times 10^7$  cells/mL. After 30 min incubation on ice, with gentle inversion, the tube was placed against a Dynal magnet (Dynal) for 2 minutes, and CD34<sup>+</sup> cells removed. Following two washes, 20  $\mu$ L of 'glycoprotease' (O-sialoglycoprotein endopeptidase, Accurate Chemical, Westbury, New York) plus 180  $\mu$ L of RPMI (JRH Biosciences)/20% FCS were added and the beads incubated at 37°C for 30 min to cleave the QBEND10 epitope, and release CD34<sup>+</sup> cells from the beads. Beads were then washed three times to maximize cell recovery. The glycoprotease used for the release step in the positive selection procedure has been shown not to effect subsequent ex vivo expansion of progenitors. Marsh et al. (1992) Leukemia 6:926.

Mobilized peripheral blood (MPB) samples were obtained with informed consent from multiple myeloma patients treated at the University of Arkansas Medical Center. The patients were treated on day 1 with cyclophosphamide at 6 g/m<sup>2</sup> (1.5 g/m<sup>2</sup> every 3 hours x 4 doses). From day 1 until the start of leukopheresis (usually 10-28 days), granulocyte macrophage colony stimulating factor (GM-CSF) was given at 0.25 mg/m<sup>2</sup>/day. Apheresis for total white cells was started when the peripheral blood white cell count was greater than 500 cells/ $\mu$ l and the platelet count was greater than 50,000 cells/ $\mu$ l. Patients were apheresed daily until from  $6 \times 10^8$  mononuclear cells (MNC) were collected.

Fresh MPB samples were elutriated with a JE5.0 Beckman counterflow elutriator equipped with a Sanderson chamber (Beckman, Palo Alto, CA). Cells were resuspended in elutriation medium (Biowhittaker, Walkersville, MD) at pH 7.2, supplemented with 0.5% human serum albumin (HSA). The rotor speed was set at 2000 RPM, the cells were introduced, and the first fraction collected at a flow rate of 9.6 ml/min. Fractions 2 and 3 were collected at the respective flow rates of 14 and 16 ml/min. The larger cells remaining in the chamber were collected after stopping the rotor. Cells were

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resuspended in RPMI supplemented with 5% HSA, 10 µg/ml DNase I and penicillin/streptomycin at 50 U/ml and 50 µg/ml, respectively. Fractions 2 and 3 were pooled and incubated with 1 mg/ml heat-inactivated human gamma-globulin to block non-specific Fc binding. Granulocytes were further depleted by incubation with CD15 conjugated to magnetic beads (Dynal M450, Oslo, Norway) followed by magnetic selection.

CD34<sup>+</sup>Thy1<sup>+</sup>LIN<sup>-</sup> cells were isolated from AMB and MPB by flow cytometry as follows. Antibodies to CD14 and CD15 were obtained as FITC conjugates from Becton-Dickinson. Antibody to Thy-1 (GM201) was obtained from Dr. Wolfgang Rettig (Ludwig Institute, New York), and was detected with anti-γ1-PE conjugate from Caltag. Antibody to CD34 (Tük 3) was obtained from Dr. Andreas Ziegler (University of Berlin), and detected with an anti-γ3-Texas Red conjugate (Southern Biotechnologies).

Anti-CD34 antibody or an IgG3 isotype matched control were added to cells in staining buffer (HBSS, 2% FCS, 10 mM HEPES) for 20 minutes on ice, together with anti-Thy-1 antibody at 5 µg/ml. Cells were washed with a FCS underlay, and then incubated with Texas Red conjugated goat anti-mouse IgG3 antibody and phycoerythrin-conjugated goat anti-mouse IgG1 antibody for 20 minutes on ice. Blocking IgG1 was then added for 10 minutes. After blocking, the FITC-conjugated lineage antibody panel (CD14 and CD15) was added, and incubated for another 20 minutes on ice. After a final washing, cells were resuspended in staining buffer containing propidium iodide (PI).

Cells were sorted on a Vantage cell sorter (Becton Dickinson) equipped with dual argon ion lasers, the primary laser emitting at 488 nm and a dye laser (Rhodamine 6G) emitting at 600 nm (Coherent Innova 90, Santa Cruz, CA) or on a high speed cell sorter as described in PCT patent application number PCT/US93/08205. Residual erythrocytes, debris and dead cells were excluded by light scatter gating plus an FL3 (PI) low gate. The sorted cell population was

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diluted 1:1 in HBSS, pelleted, and resuspended in HBSS for hemocytometer counting.

The CD34<sup>+</sup>Thy-1<sup>+</sup>LIN<sup>-</sup> cells were seeded into either 48- or 96-well tissue culture dishes, at the initial inoculation density, with cytokines at the concentrations and for the amount of time and O<sub>2</sub> concentration as indicated in each example. The cultures were not fed during the experiments, but remained in the medium into which they were initially inoculated. Unless otherwise indicated, the cells were incubated at 37°C, 5% CO<sub>2</sub> and the volume removed for cell-counting replaced with fresh media (8% volume/cell count). The media, cytokines and additives were as described above; the SSP media contained the preferred amounts of additives indicated for each medium (e.g. SSP12, SSP14, etc.).

At the end of the culture period, cells were harvested and total cells determined by hemacytometer counting. In addition, the cells were stained with sulforhodamine-conjugated αCD34 and propidium iodine and analyzed on a FACSCAN (Becton Dickinson, San Jose, CA) to determine the number of CD34<sup>+</sup> viable cells.

#### Example 2

##### Expansion of ABM CD34<sup>+</sup>Thy-1<sup>+</sup>LIN<sup>-</sup> cells in various media

The ABM CD34<sup>+</sup>Thy-1<sup>+</sup>LIN<sup>-</sup> cells were obtained as described in Example 1 and inoculated into one of three media formulations in 48 well plates. The inoculate was 10,000 cells in 0.5 mL of medium. Every medium contained the cytokines IL3 (10 ng/mL), IL6 (10 ng/mL), GM (10 ng/mL), and Flk2 (50 ng/mL). The different media used were IMDM with 10% FBS, SSP16 with 30 μL pituitary extract, and SSP16. The cells were incubated for 6 days in 5% O<sub>2</sub> and the numbers of total cells and CD34<sup>+</sup> cells were determined as described in Example 1.

The results obtained are depicted in Figure 1 where total cell growth is shown by the clear areas and total CD34<sup>+</sup> cell growth is shown by the hatched areas. The results obtained



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indicate that both SSP media resulted in greater expansion of CD34<sup>+</sup> cells than IMDM with FBS, and SSP16 with pituitary extract allowed greater total cell growth than IMDM with FBS.

### Example 3

#### Expansion of MBP CD34<sup>+</sup>Thy-1<sup>+</sup>LIN<sup>-</sup> cells in various media

The MPB CD34<sup>+</sup>Thy-1<sup>+</sup>LIN<sup>-</sup> cells were obtained as described in Example 1 and inoculated into one of three media formulations in 96 well plates. The inoculate was 1,000 cells in 0.1 mL of medium. Every medium contained the cytokines IL3 (10 ng/mL), IL6 (10 ng/mL), GM (10 ng/mL), and Flk2 (50 ng/mL). The different media used were IMDM with 10% FBS, SSP16 and SSP15. The cells were incubated for 5 or 7 days in 5% O<sub>2</sub> and the numbers of total cells and CD34<sup>+</sup> cells were determined as described in Example 1.

The results obtained are depicted in Figures 2A (5 day incubation) and 2B (7 day incubation). In Figure 2, total cell growth is shown by the clear areas and total CD34<sup>+</sup> cell growth is shown by the hatched areas. The results obtained indicate that for both iterations of SSP medium, there were more total proliferation and retention of CD34<sup>+</sup> phenotype at both 5 and 7 days compared to IMDM with FBS.

In order to determine if the higher proliferation rate could be maintained over time, MPB CD34<sup>+</sup>Thy-1<sup>+</sup>LIN<sup>-</sup> cells were obtained as described in Example 1 and inoculated into one of three media formulations in 48 well plates. The inoculate was 1,000 cells in 0.1 mL of medium. Every medium contained the cytokines IL3 (10 ng/mL), IL6 (10 ng/mL), GM (10 ng/mL), and Flk2 50 (ng/mL). The different media used were IMDM with 10% FBS, SSP16 and SSP15. The cells were incubated for 13 days in 5% O<sub>2</sub> and the number of CD34<sup>+</sup> cells were determined as described in Example 1.

The results obtained are depicted in Figure 3 and indicate that the higher proliferation rate for the two SSP media were maintained for 13 days.

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## Example 4

Effect of c-kit on ABM CD34<sup>+</sup>Thy-1<sup>-</sup>LIN<sup>-</sup> cell expansion

The ABM CD34<sup>+</sup>Thy-1<sup>-</sup>LIN<sup>-</sup> cells were obtained as described in Example 1 and inoculated into 48 well plates in one of two media formulations: IMDM with 10% FBS or SSP16 with KL (50 ng/mL). Each medium contained the cytokines IL3 (10 ng/mL), IL6 (10 ng/mL), GM (10 ng/mL), and Flk2 (50 ng/mL). The inoculates were either 2,000 cells in 0.1 mL of medium or 1,400 cells in 0.1 mL of medium. The cells were incubated for 5 days in 5% O<sub>2</sub> and the numbers of total cells and CD34<sup>+</sup> cells were determined as described in Example 1.

The results obtained are depicted in Figures 4A (2000 cells/100  $\mu$ L inoculate) and 4B (1400 cells/100  $\mu$ L inoculate). In Figure 4, total cell growth is shown by the clear areas and total CD34<sup>+</sup> cell growth is shown by the hatched areas. The results showed that the addition of c-kit ligand (KL) to IL3, IL6, GM, and Flk2 ligand to SSP medium, enhanced total cell proliferation and maintenance CD34<sup>+</sup> phenotype in the SSP media.

## Example 5

Effect of O<sub>2</sub> on ABM CD34<sup>+</sup>Thy-1<sup>-</sup>LIN<sup>-</sup> cell expansion

The ABM CD34<sup>+</sup>Thy-1<sup>-</sup>LIN<sup>-</sup> cells were obtained as described in Example 1 and inoculated into 96 well plates in one of two media formulations: IMDM with 10% FBS or SSP16 with KL (50 ng/mL). Each medium contained the cytokines IL3 (10 ng/mL), IL6 (10 ng/mL), GM (10 ng/mL), and Flk2 (50 ng/mL). The inoculates were 2,000 cells in 0.1 mL of medium. The cells were incubated for 5 days in 5% O<sub>2</sub> or 20% O<sub>2</sub> and the numbers of total cells and CD34<sup>+</sup> cells were determined as described in Example 1.

The results obtained are depicted in Figures 5A (5% O<sub>2</sub>) and 5B (20% O<sub>2</sub>). In Figure 5, total cell growth is shown by the clear areas and total CD34<sup>+</sup> cell growth is shown by the hatched areas. The results showed that both the total cell and CD34<sup>+</sup> cell expansions were higher in the SSP formula

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compared to IMDM with FBS and were higher in 20% O<sub>2</sub> compared to 5% O<sub>2</sub>.

#### Example 6

##### ABM CD34<sup>+</sup>Thy-1<sup>+</sup>LIN<sup>-</sup> cell expansion without cytokines

CD34<sup>+</sup>Thy-1<sup>+</sup>LIN<sup>-</sup> cells were obtained from two different bone marrow donors according to the method described in Example 1 and inoculated into 96 well plates in one of two media formulations: IMDM with 10% FBS or SSP16. Both media were supplemented with Flk2 (50 ng/mL) only, neither contained the cytokines IL3, IL6, GM or KL. The inoculates were 2,000 cells in 0.1 mL of medium. The cells were incubated for 6 days in 5% O<sub>2</sub> and the numbers of total cells and CD34<sup>+</sup> cells were determined as described in Example 1.

The results obtained are depicted in Figures 6A (donor #11281) and 6B (donor #11279). In Figure 6, total cell growth is shown by the clear areas and total CD34<sup>+</sup> cell growth is shown by the hatched areas. The results showed both that the addition of cytokines is necessary to the SSP media to obtain optimal cellular expansion and that the results are reproducible with different samples of bone marrow.

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and the examples are intended to illustrate and not limit the scope of the invention. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

## Claims

1. A composition comprising a serum-free medium comprising basal medium, effective amounts of at least one of the following: an autoclavable peptone, a protease inhibitor and a pituitary extract or a purified factor thereof, and, optionally, effective amounts of at least one of the following: serum albumin or plasma protein fraction, an anti-clotting compound, a reducing agent, insulin, transferrin and ethanolamine or phosphoethanolamine, low density lipoprotein or human cholesterol concentrate, linoleic acid, oleic acid, vitamin A, vitamin E, antioxidant, ascorbic acid, a polyamine, vitamin B12 and a steroid hormone.

2. The composition according to claim 1 wherein the basal medium is IMDM.

3. The composition according to claim 1 wherein the basal medium is SSP.

4. The composition according to claim 1, wherein the peptone is selected from the group consisting of meat peptone, soy bean peptone and mixed peptones.

5. The composition according to claim 4, wherein the peptone is meat peptone and is present in an amount of about 0.05 to 0.35 percent of the final solution.

6. The composition according to claim 1, wherein the protease inhibitor is selected from the group consisting of aprotinin, catalase and superoxide dismutase.

7. The composition according to claim 6, wherein the protease inhibitor is aprotinin and present in an amount of about 2,000 to 20,000 KIU units/L.

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8. The composition according to claim 1, wherein the pituitary extract is present in an amount of about 3 mg/L to 75 mg/L.

9. The composition according to claim 1 wherein the pituitary purified factor is selected from the group consisting of activin, inhibin, heregulin and growth hormone.

10. The method according to claim 1 wherein the serum albumin is selected from the group consisting of human albumin, bovine serum albumin and equine serum albumin.

11. The composition according to claim 10, wherein the serum albumin is human and is present in an amount of about 1 to 3 percent of the final solution.

12. The composition according to claim 1, wherein the plasma protein fraction is present in an amount of about 1 to 4 percent.

13. The composition according to claim 1, wherein the heparin is present in an amount of about 5,000 to 40,000 USP units/L.

14. The composition according to claim 1, wherein the reducing agent is selected from the group consisting of  $\beta$ -mercaptoethanol, thiols, aminoguanine, 3-hydroxybenzyloxy amines and low  $O_2$  tension.

15. The composition according to claim 14, wherein the reducing agent is  $\beta$ -mercaptoethanol, and is present in an amount of from about 50 to 300  $\mu$ M.

16. The composition according to claim 1, wherein the insulin is present in an amount of from about 2 to 100 mg/L.

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17. The composition according to claim 16, wherein the insulin is present in an amount of about 20 mg/L.

18. The composition according to claim 1, wherein the transferrin is present in an amount of from about 2 to 400 mg/L.

19. The composition according to claim 1, wherein the ethanolamine is present in an amount of from about 22 to 11 mg/L.

20. The composition according to claim 1, wherein the low density lipoprotein is present in an amount of from about 1 to 10 mg/L.

21. The composition according to claim 1, wherein the human cholesterol concentrate is present in an amount of from about 2.13 to 340.48 mg/L.

22. The composition according to claim 1, wherein the linoleic acid is present in an amount of from about  $10^{-4}$  to  $10^{-7}$  M.

23. The composition according to claim 1, wherein the oleic acid is present in an amount of from about  $10^{-4}$  to  $10^{-7}$  M.

24. The composition according to claim 1, wherein the vitamin A is present in an amount of from about  $10^{-4}$  to  $10^{-7}$  M.

25. The composition according to claim 1, wherein the vitamin E is present in an amount of from about  $10^{-4}$  to  $10^{-7}$  M.

26. The composition according to claim 1, wherein the antioxidant is superoxide dismutase and is present in an amount of from about 2,000 to 20,000 units/L.

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27. The composition according to claim 1, wherein the ascorbic acid is present in an amount of from about  $10^{-3}$  to  $10^{-7}$  M.

28. The composition according to claim 1, wherein the polyamine is selected from the group consisting of putrescine, spermine and spermidine.

29. The composition according to claim 28, wherein the polyamine is putrescine and is present in amount of from about  $10^{-5}$  to  $10^{-9}$  M.

30. The composition according to claim 1, wherein the vitamin B12 is present in an amount of from about  $10^{-4}$  to  $10^{-8}$  M.

31. The composition according to claim 1 wherein the steroid hormone is selected from the group consisting of progesterone, estrogen, androgen, testosterone and effective analogs thereof.

32. The composition according to claim 31, wherein the steroid hormone is progesterone and is present in an amount of from about  $10^{-7}$  to  $10^{-13}$  M.

33. The composition according to claim 31, wherein the steroid hormone is testosterone and is present in an amount of from about  $10^{-6}$  to  $10^{-12}$  M.

34. The composition according to claim 1, further comprises at least one cytokine.

35. The composition according to claim 34, wherein the at least one cytokine is selected from the group consisting of IL3, IL6, c-kit ligand, GM-CSF, G-CSF and flk2/flt3 ligand.

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36. The composition according to claim 1, wherein the osmolality is titrated with NaCl to from about 260 to 320 mOsm.

37. The composition according to claim 1, further comprising an antibiotic.

38. The composition according to claim 37, wherein the antibiotic is polymyxin B sulfate in an amount of from 10 µg/L to 100 µg/L.

39. The composition according to claim 1, further comprising hematopoietic progenitor and/or stem cells.

40. The composition according to claim 1, further comprising a cryopreservative.

41. The composition according to claim 40, wherein the cryopreservative is dimethyl sulfoxide.

42. The composition according to claim 41, further comprising Hetastarch™, pentastarch or methylcellulose.

43. The composition according to claim 42 wherein Hetastarch™ is present in an amount of from about 1 to 4 percent.

44. The composition according to claim 40, further comprising hematopoietic and/or stem cells.

45. A composition comprising a serum-free medium comprising SSP, a peptone, a protease inhibitor and an antioxidant and effective amounts of at least one of the following: human serum albumin or plasma protein fraction, heparin, a reducing agent, insulin, transferrin and ethanolamine.



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46. The composition according to claim 45 wherein the peptone is a meat peptone, the protease inhibitor is aprotinin and the antioxidant is SOD.

47. The composition according to claim 45, further comprising effective amounts of low density lipoprotein or human cholesterol concentrate, linoleic acid, oleic acid, vitamin A and vitamin E.

48. The composition according to claim 47, further comprising effective amounts of putrescine, vitamin B12 and at least one steroid hormone.

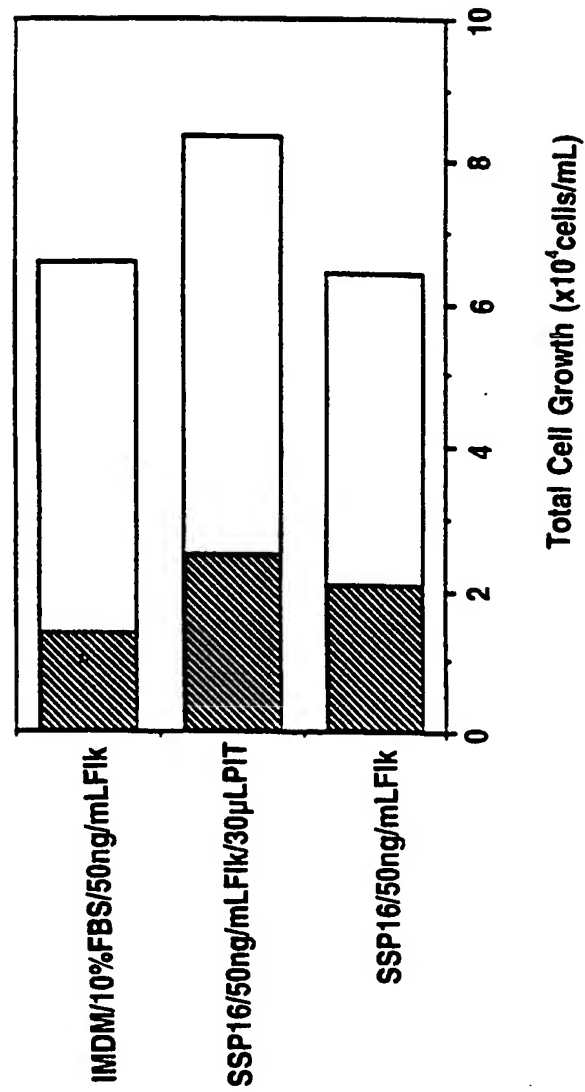
49. The composition according to claim 48 wherein the steroid hormone is at least one of progesterone, androgen, estrogen, testosterone or physiologically effective analogs thereof.

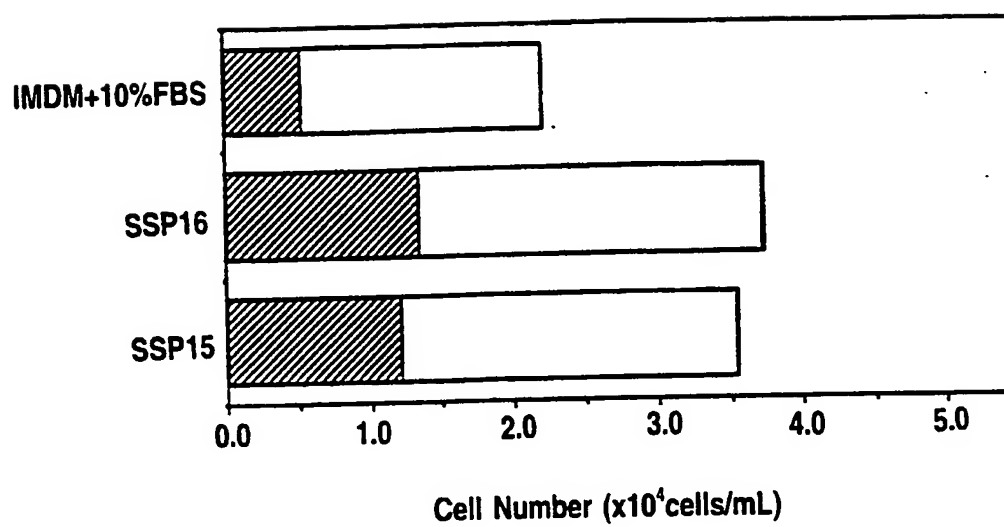
50. Use of a composition according to any of claims 1-49 in the propagation of human hematopoietic cells.

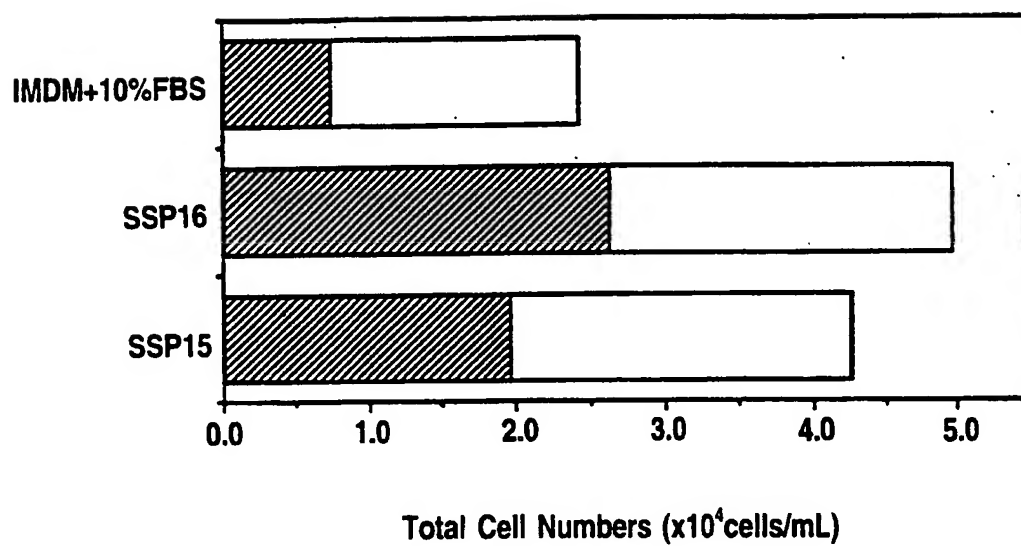
51. The use according to claim 50 when the cells are human hematopoietic stem cells.

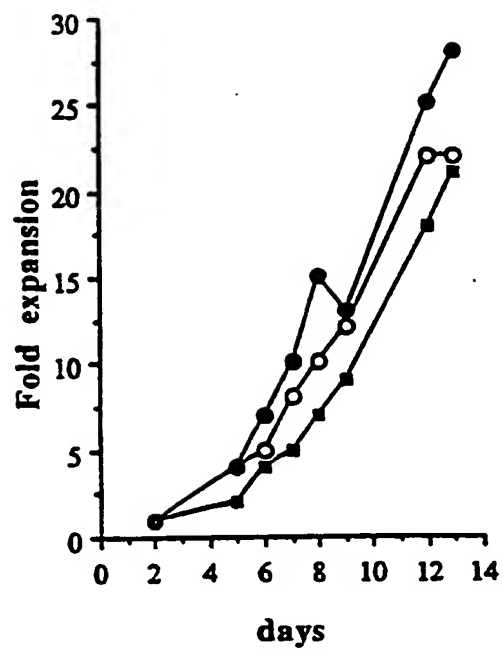
52. A method of producing human hematopoietic cells comprising propagating said cells in a composition according to any of claims 1-49 and recovering the cells thus propagated.

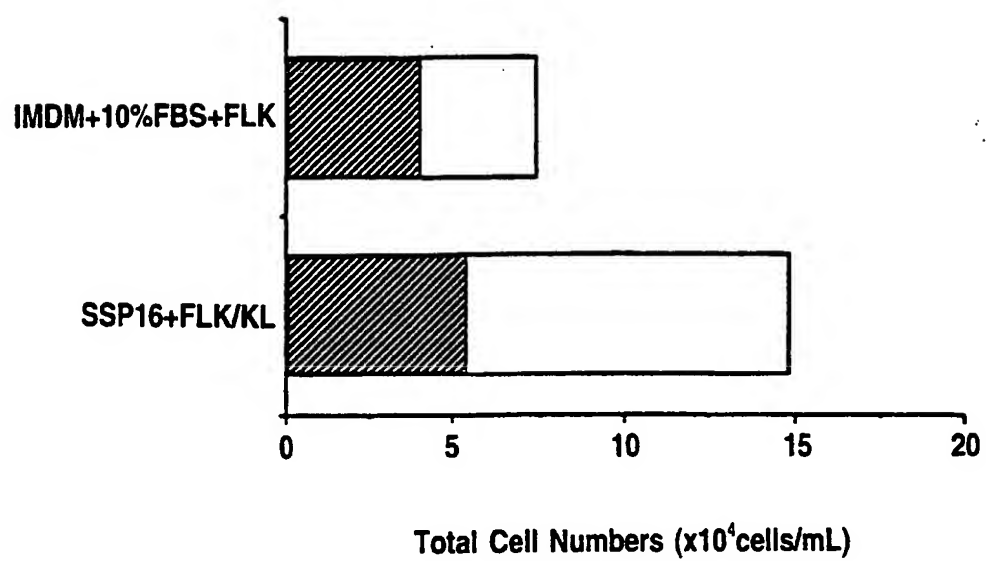
53. The method according to claim 52 wherein the human hematopoietic cells are human hematopoietic stem cells.

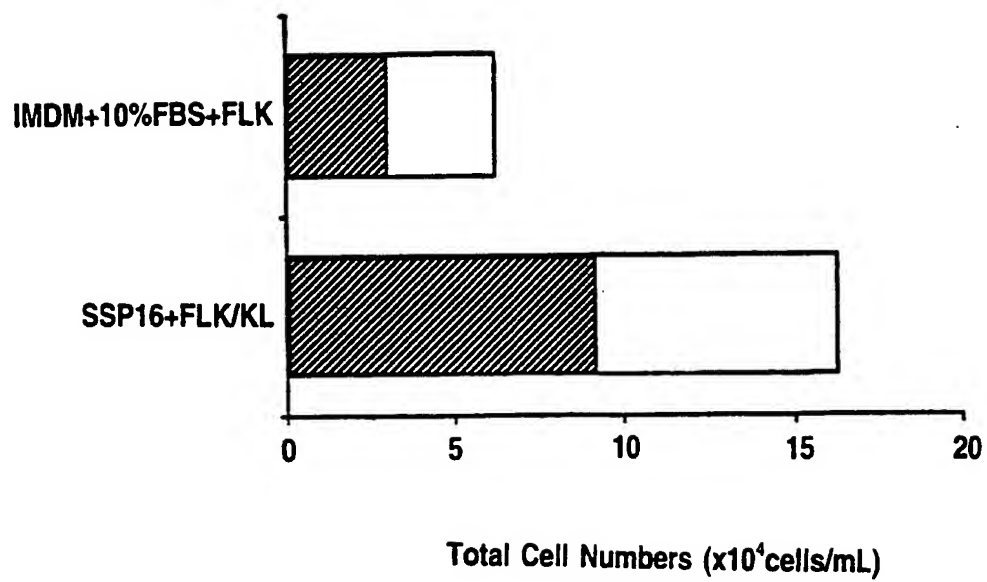
**Figure 1**

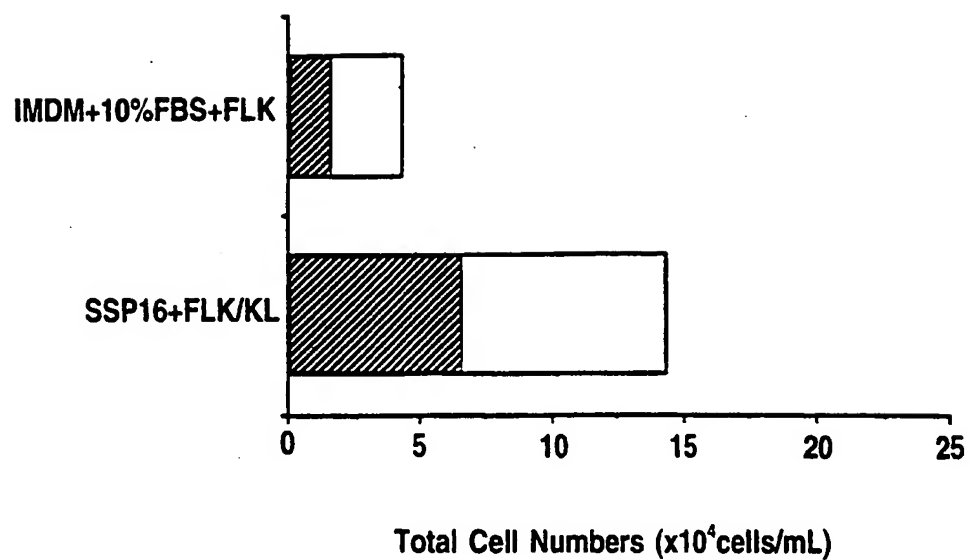
**Figure 2A**

**Figure 2B**

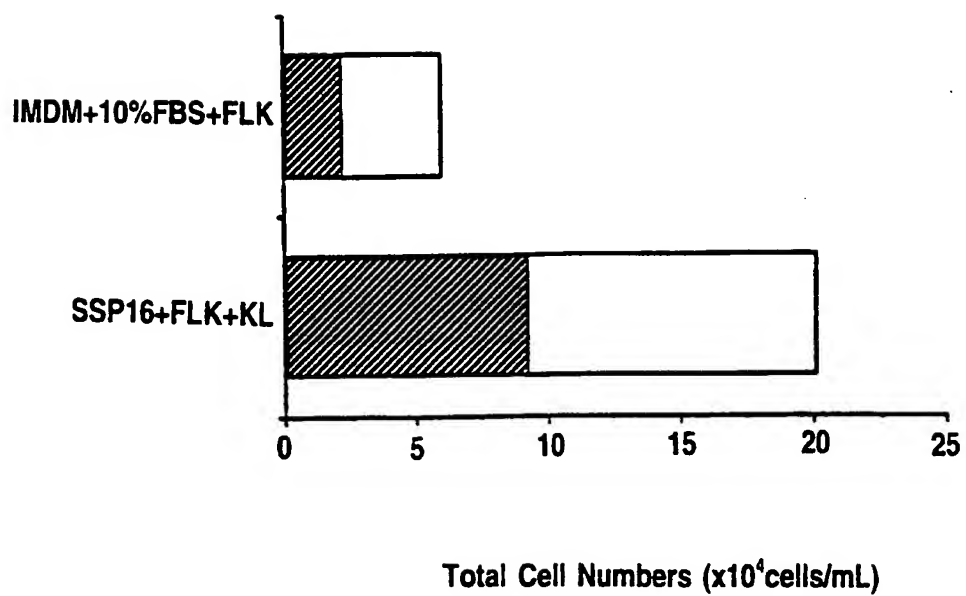
**Figure 3**

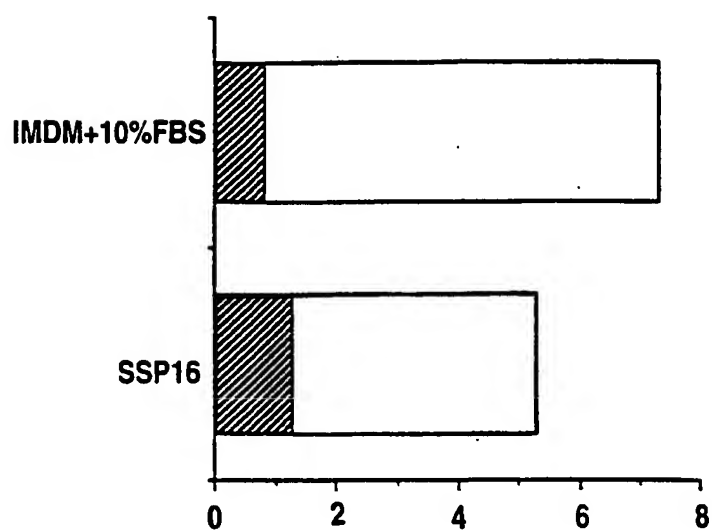
**Figure 4A**

**Figure 4B**

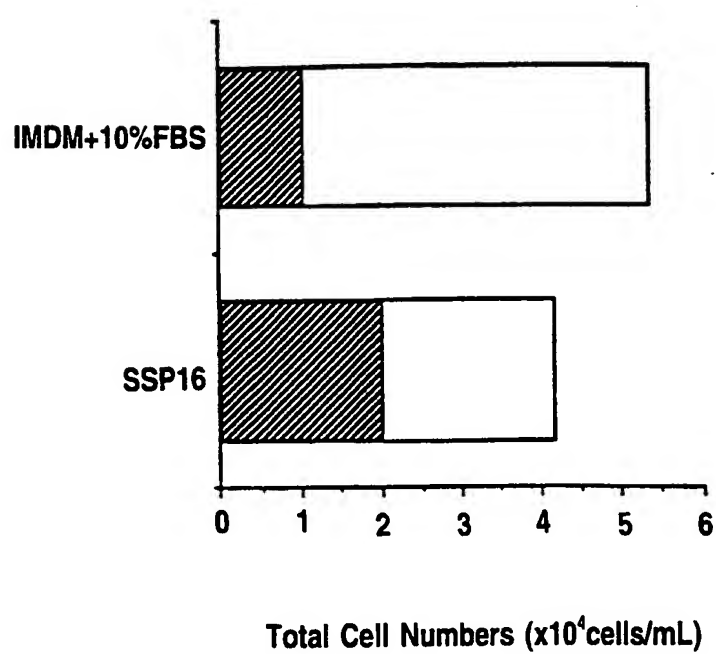
**Figure 5A**



**Figure 5B**

**Figure 6A**

Total Cell Numbers (x10<sup>4</sup>cells/mL)

**Figure 6B**

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 96/02454

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO,A,95 29231 (GENZYME CORP) 2 November 1995 see the whole document ---	1
X	EP,A,0 550 760 (FUJI YAKUHI KOGYO KK) 14 July 1993  see the whole document ---	1-3,10, 11, 16-18, 27-30, 44,50,52
X	R. IAN FRESHNEY: "CULTURE OF ANIMAL CELLS-A MANUAL OF BASIC TECHNIQUE" 1987 , ALAN R. LISS, INC. , NEW YORK XP002014579  see page 74 - page 84 ---	1-3, 8-12, 14-19, 21,25, 30,31
-/--		

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

27 September 1996

Date of mailing of the international search report

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## INTERNATIONAL SEARCH REPORT

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A	WO,A,95 00632 (AMGEN INC) 5 January 1995 see the whole document -----	1-53

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